(FIG. 8), respectively, and were similar in structure to the *cryI* plasmids described in U. S. Patent 5,441,884 (specifically incorporated herein by reference).

Strain EG4923-4 transformants containing plasmids pEG348 and pEG1641 were isolated on Luria plates containing 10 μg/ml tetracycline. Recombinant plasmid DNAs from the transformants were isolated by the alkaline lysis procedure described by Baum (1995) and confirmed by restriction enzyme analysis. The plasmid arrays of the transformants were further confirmed by the Eckhardt agarose gel analysis procedure described by Gonzalez Jr. *et al.*, (1982). The EG4923-4 recombinant derivatives were designated EG4923-4/pEG348 and EG4923-4/pEG1641.

5.8 EXAMPLE 8 -- MODIFICATION OF EG4923-4/pEG348 AND EG4923-4/pEG1641 TO REMOVE FOREIGN DNA ELEMENTS

pEG348 and pEG1641 contain duplicate copies of a site-specific recombination site or internal resolution site (IRS) that serves as a substrate for an *in vivo* site-specific recombination reaction mediated by the TnpI recombinase of transposon Tn*5401* (described in Baum, 1995). This site-specific recombination reaction, described in U. S. Patent 5,441,884, results in the deletion of non-*B. thuringiensis* DNA or foreign DNA elements from the crystal protein-encoding recombinant plasmids. The resulting recombinant *B. thuringiensis* strains are free of foreign DNA elements, a desirable feature for genetically engineered strains destined for use as bioinsecticides for spray-on application. Strains EG4923-4/pEG348 and EG4923-4/pEG1641 were modified using this *in vivo* site-specific recombination (SSR) system to generate two new strains (Table 12), designated EG7841-1 (alias EG11730) and EG7841-2 (alias EG11831). The recombinant plasmids in strains EG7841-1 and EG7841-2 were designated pEG348Δ and pEG1641Δ, respectively.

TABLE 12

RECOMBINANT *B. THURINGIENSIS* STRAINS

Strain	Alias	Recombinant plasmid	Progenitor strain
EG7841-1	EG11730	pEG348Δ	EG4923-4/pEG348
EG7841-2	EG11831	pEG1641Δ	EG4923-4/pEG1641

EXAMPLE 9 -- CRY1C COMBINATORIAL MUTANTS AT AA POSITIONS 148 AND 219

The *cry1C-R148A* gene on pEG1639 and the *cry1C-R148D* gene on pEG1642 were used as templates for additional mutagenesis studies aimed at achieving further improvements in insecticidal activity.

In one example, the lysine residue at position 219 (K219) was replaced with an alanine residue, using the PCR™-based mutagenesis protocol described by Michael (1994) and the mutagenic oligonucleotide primer J:

Primer J: (SEQ ID NO:62)

5'-CGGGGATTAAATAATTTACCGGCTAGCACGTATCAAGATTGGATAAC-3'

Primer J also incorporates a unique *Nhe*I site (underlined above) that can be used to distinguish the original gene from the mutant gene by restriction enzyme analysis. The PCR™-mediated mutagenesis reactions employed the flanking primers H (SEQ ID NO:52) and F (SEQ ID NO:20), the mutagenic oligonucleotide primer J (SEQ ID NO:62), and pEG1639 (*cry1C-R148A*) as a template. In these reactions, 5 units of *Taq* Extender™ (Stratagene) were included to improve the efficiency of amplification with *Taq* polymerase. The amplified products from the mutagenesis reaction were resolved by agarose gel electrophoresis and the amplified DNA fragment incorporating the mutagenic oligonucleotide primer J was excised from the gel and purified using the Geneclean II® procedure. This DNA fragment was cleaved with the restriction endonucleases *Bbu*I and *Age*I.

In order to subclone the *Bbu*I-*Age*I *cry1C* restriction fragment and express the mutant *cry1C* gene in *B. thuringiensis*, the *cry1C* plasmid pEG345 (FIG. 3) was cleaved with *Bbu*I and *Age*I, treated with calf intestinal alkaline phosphatase (Boehringer

Mannheim Corp.), and the resulting DNA fragments resolved by agarose gel electrophoresis. The larger vector fragment was excised from the gel and purified using the Geneclean II® procedure. The pEG345 vector fragment was subsequently ligated to the amplified *cry1C* fragment recovered from the mutagenesis reaction and the ligation products used to transform *E. coli* Sure™ cells (Stratagene) to ampicillin resistance using electroporation. Individual colonies recovered from Luria plates containing 50 µg/ml ampicillin were isolated and inoculated into 3 ml cultures containing 1X brain heart infusion, 0.5% glycerol (BHIG), and 50 µg/ml ampicillin.

Plasmid DNAs were prepared from the broth cultures using the alkaline lysis method, digested with the restriction enzyme *Nhe*I, and resolved by agarose gel electrophoresis to distinguish clones incorporating the mutagenic sequence of primer J and therefore encoding the alanine substitution at position 219. Incorporation of the mutant sequence into *cry1C-R148A* was confirmed by DNA sequence analysis. Plasmid DNAs from four recombinant *E. coli* clones were used to transform the acrystalliferous *B. thuringiensis* strain EG10368 to chloramphenicol resistance using electroporation. Transfer of the recombinant plasmid to EG10368 was confirmed by restriction enzyme analysis of plasmid DNAs recovered from the EG10368 transformants. One chloramphenicol resistant colony was selected and designated EG12111. The *cry1C* gene in EG12111 was designated *cry1C-R148A K219A* (SEQ ID NO:58) and the encoded crystal protein designated Cry1C-R148A K219A (SEQ ID NO:59).

The same substitution was made in Cry1C-R148D using the same procedures but using pEG1642 (*cry1C-R148D*) as the template for the PCR™-mediated mutagenesis reaction. The ligation products were used to transform *E. coli* DH5α cells to ampicillin resistance using standard transformation procedures. Plasmid DNAs were prepared from broth cultures of selected ampicillin resistant clones using the alkaline lysis method, digested with the restriction enzyme *Nhe*I, and resolved by agarose gel electrophoresis to distinguish clones incorporating the mutagenic sequence of primer J and therefore encoding the alanine substitution at position 219. Incorporation of the mutant sequence into *cry1C-R148D* was confirmed by DNA sequence analysis. Recombinant plasmids from three mutant clones were used to transform the acrystalliferous *B. thuringiensis*